Novel Markers for Liquid Biopsies in Cancer Management: Circulating Platelets and Extracellular Vesicles

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ABSTRACT

Although radiologic imaging and histologic assessment of tumor tissues are classic approaches for diagnosis and monitoring of treatment response, they have many limitations. These include challenges in distinguishing benign from malignant masses, difficult access to the tumor, high cost of the procedures, and tumor heterogeneity. In this setting, liquid biopsy has emerged as a potential alternative for both diagnostic and monitoring purposes. The approaches to liquid biopsy include cell-free DNA/circulating tumor DNA, long and micro noncoding RNAs, proteins/peptides, carbohydrates/lectins, lipids, and metabolites. Other approaches include detection and analysis of circulating tumor cells, extracellular vesicles, and tumor-activated platelets. Ultimately, reliable use of liquid biopsies requires bioinformatics and statistical integration of multiple datasets to achieve approval in a Clinical Laboratory Improvement Amendments setting. This review provides a balanced and critical assessment of recent discoveries regarding tumor-derived biomarkers in liquid biopsies along with the potential and pitfalls for cancer detection and longitudinal monitoring.

Introduction

Routine clinical practice has relied on tissue sampling to diagnose tumors (1, 2). Despite the tremendous informative value of high-quality tumor biopsies, there are many limitations to their use. Tissue biopsies are invasive, costly, and a possible source of clinical complications. Adverse events such as air leaks in the chest, postprocedural hypotension, and hemorrhage occur in 1.6% to 17.1% of cases (3). Moreover, tissue preservation techniques (formalin fixation and paraffin embedding, snap freezing, and gel-preserved freezing; ref. 4) can distort the original quality of samples that were preserved for years. Liquid nitrogen–based snap freezing and more elaborate preservation methods are typically not available in a community hospital setting. Moreover, focal tissue sampling is not always able to capture the heterogeneity of the tumor microenvironment since biopsy cores cannot sample the entire tumor region. Finally, there are cases in which the primary site can be challenging to identify on imaging, or the tumor mass is localized in an area that is difficult to access, making it difficult to obtain a biopsy. Because of these and other limitations, the medical community has sought to produce and standardize noninvasive techniques that can be safely performed longitudinally and complement or substitute invasive approaches. Because liquid biopsies drawn from the entire tumor vascular drainage, this approach may be more reflective of the primary tumor and its collective metastases.

Because of their minimal invasiveness, liquid biopsies can be repeated and provide biological material that often requires minimal efforts for preservation (5), making this potentially one of the most useful techniques for early diagnosis, monitoring of therapy response, and prediction of prognosis. To date, several tumor-derived liquid biopsy biomarkers have been developed, including circulating tumor cells (CTC), extracellular vesicles (EV) or exosomes, cell-free (cfDNA) or circulating tumor DNA (ctDNA), epigenetically modified DNA, long and micro noncoding RNAs (ncRNA), and platelets (**Fig. 1**). Isolation of ctDNA and circulating cancer cells is typically influenced by disease burden and cancer type (6, 7). ctDNA is used to detect tumor-specific mutations or epigenetic modifications for genotyping metastasis (8), minimal residual disease (9), and staging for treatment decisions (10–12). The EVs from tumor cells can contain mRNAs, proteins, and other potential biomarkers (13). Platelets supply considerable amount of EVs to the circulation and also show robust uptake through their open canalicular system (14, 15). Moreover, platelets are among the easiest biological particles to isolate and can provide a complete molecular profile of their passage through a tumor (ref. 16; **Fig. 1**).

Ultimately, effective liquid biopsies depend on the integration of multiple biomarkers, sampling approaches, and datasets. This results in a highly sensitive and specific representation of the disease status (17–19).

Liquid Biopsies: Principal Biomarkers

Tumor-derived factors

cf/ctDNA and CTCs

The presence of cfDNA in plasma and serum was first reported in 1948 by Mandel and Metais (20) but remained largely unexplored until the 1970s; since then it has drawn substantial attention for clinical use as a noninvasive biopsy (21). One of its most common applications in clinical practice is the analysis of circulating cfDNA to assess abnormal fetal conditions in high-risk pregnancies, such as trisomy of chromosomes 21, 18, or 13 (22). This technique, usually performed with high patient compliance, can be used as early as the 10th week of pregnancy until delivery and has almost completely replaced invasive diagnostic techniques such as amniocentesis.

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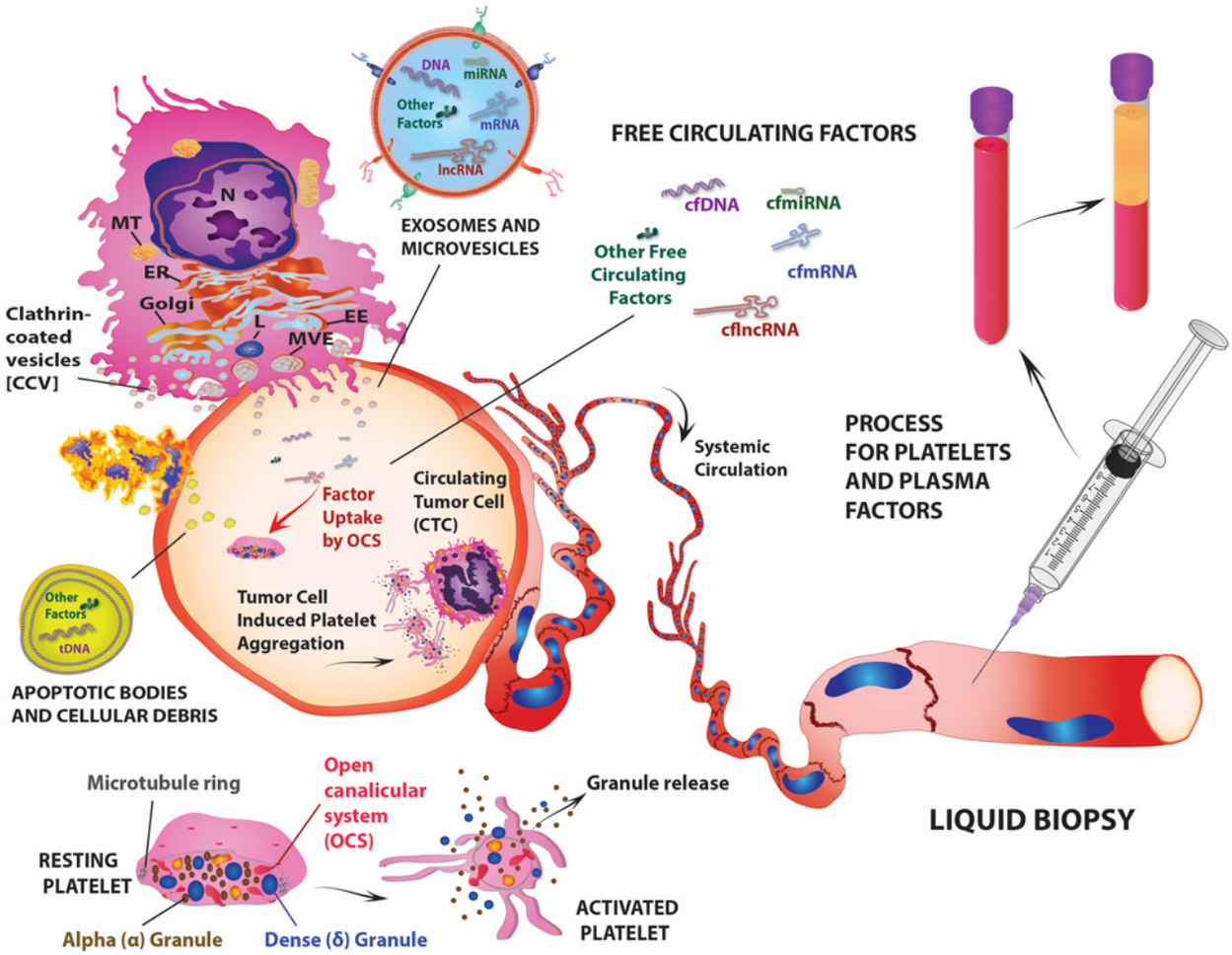

Figure 1.
The illustration represents the "Circulome" consisting of the different circulating factors derived from or influenced by cancer cells. MT: mitochondria; N: nucleus; ER: endoplasmic reticulum; MVE: multi-vesicular endosome; EE; early endosome; cf; circulating-free; tDNA: transfer DNA; miRNA: microRNA; mRNA: messenger RNA; lncRNA: long noncoding RNA.

DNA can be released into the bloodstream at any point from primary tumor cells, CTCs, or tumor-derived EVs. cfDNA typically consists of any circulating DNA molecule in the bloodstream, not necessarily cancer derived. In contrast, ctDNA is specifically tumor derived. Regardless of its source, cfDNA (or ctDNA) is actively secreted or passively released into the bloodstream by tumor cells undergoing apoptosis, necrosis (23), or immunotherapy-associated tumor killing (24–26). Some studies have shown concordance between ctDNA and DNA isolated from the original tumor tissue (27–29). Sequencing analyses have revealed similar mutations, copy-number variations, and gene amplification patterns between the ctDNA and DNA of tumor tissues (both primary and metastatic). However, other studies have found substantial rates of discordance between ctDNA and tumor DNA in identifying specific mutations. In a study on a cohort of patients with lung cancer ($N = 38$), a comparison between four platforms for mutation detection on ctDNA showed concordance between tumor tissue and ctDNA in detecting the EGFR T90M mutation ranging between 48% and 74% (30). In a heterogeneous cohort of 74 patients with different cancer types, concordance for detection of relevant alterations in 33 genes with microsatellite instability between cfDNA and matched tumor samples was tested. There was only 45% concordance when considering primary tumor samples (77% when considering DNA from metastatic disease; ref. 31). The discordant rates are often related to low variant allele frequency or intratumoral heterogeneity (32, 33).

Some studies have suggested that ctDNA analysis can complement DNA analysis from primary and metastatic tumor tissue biopsies when investigating TP53 mutations and other repair genes (34). A potential limitation of standard DNA-sequencing assays (e.g., next-generation sequencing) applied to ctDNA analyses (29) is characterized by the observation of a lower number of mutation reads, due to the small amount of tumor-specific DNA. To address this issue, more specific detection techniques have been developed, such as digital droplet PCR and several commercial assays for ctDNA amplification are being used in the clinical setting for various cancers (10, 11, 35). The short half-life in circulation, fragment size, and available volume are critical considerations when designing and evaluating ctDNA analysis approaches for plasma samples (8, 36, 37). Recently Natera's Signatera (TM) MRD (molecular residual disease) test which uses ctDNA, has been granted Breakthrough Device Designation (38).

FDA-approved commercial assays for detection of mutations in circulating cancer-derived DNA include FoundationOne Liquid CDx [for non–small cell lung cancer (NSCLC) and prostate cancer] and Guardant360 CDx (for NSCLC and colorectal cancer), as companion diagnostic tests. While FoundationOne targets coding regions of 324 genes and among these, 72 genes with higher depth (39), the Guardant360 assay targets 54 genes, with complete exon coverage of 18 and coverage of critical regions of 36 genes (including ERBB2, EGFR, and MET; ref. 40).

Another opportunity for liquid biopsies involves CTCs that are cancer cells shed from the tumor into the bloodstream (41). Several studies have shown a high rate of concordance in gene mutations between circulating tumor cells and DNA from tumor tissue, making CTCs a potentially suitable candidate as a cancer biomarker (42–44). Clinically, the abundance of CTCs has been associated with a worse prognosis in prostate, lung, and other cancers. These clinical observations are corroborated by experimental findings that have demonstrated the potential of CTCs to form metastatic clusters (45). However, their role as a diagnostic marker remains unclear due, in part, to the lack of a specific marker for detection of all CTCs (46). A recent study in support of their diagnostic potential, including 112 patients with hepatocellular carcinoma, 20 healthy donors, and 12 patients with hepatitis B virus, reports detection of CTCs in 90% of the patients with hepatocellular carcinoma and in 17% of the patients with hepatitis B who further developed hepatocellular carcinoma (47). CTCs were proven to originate from the primary tumor, as suggested by identifying specific TP53 DNA mutations that matched the ones isolated in the primary tumor DNA. In addition, specific markers on CTCs (e.g., PD-L1) could be useful as a marker for response to therapy, as a recent pilot study on the efficacy of pembrolizumab in patients with advanced melanoma shows (48).

Despite their potential, CTCs are rare in the bloodstream [some studies report between one and six cells in 7.5 mL of whole blood (49)], and can be challenging to isolate. Up to now, the only FDA-approved method for CTC isolation has been the Cell Search System (Jenssen Diagnostics), which uses anti–EpCam-coated magnetic beads; this method has been used successfully in clinical trials for prognostic stratification. Several new techniques have since been tested to isolate higher quality material and avoid the bias of *a priori* knowledge of surface markers (needed for antibody-based isolation techniques). For example, acoustic separation uses the physical properties of CTCs to separate them from other circulating cells (50). Utilizing marker-directed antibodies for positive selection of cells has the apparent disadvantage of selecting just a specific subpopulation of circulating tumor-derived cells. Many tumor epithelial cells can express low levels of EpCAM or be entirely negative for it and positive for other cytokeratins (51). Besides that, many circulating cells have likely undergone epithelial–mesenchymal transition (EMT) with consequent loss of epithelial markers, which justifies the use of negative selection for CD45, without positive selection for cytokeratins (52). Although newer approaches, such as coupling CTC and ctDNA isolation to single-cell sequencing, may hold promise in the future (53, 54), the low specificity and high dilution represent the major drawbacks of using these currently in routine clinical practice. Finally, a specific phenomenon termed "clonal hematopoiesis of indeterminate potential" (55), may affect the reliability of DNA-sequencing data from cfDNA/ctDNA. Clonal hematopoiesis (CH) represents age-related acquisition of somatic mutations in hematopoietic cells, which leads to clonal expansion and potentially to hematopoietic diseases (56). Its incidence is quite high in the aging population, reaching 10% of healthy subjects after the age of 70 (57). This somatic mosaicism present in plasma could lead to misclassification of CH mutations as tumor derived and represents an important source of background noise which some have tried to control for using bioinformatics.

To overcome these limitations, scientists have investigated the presence of more specific tumor-derived circulating tumor biomarkers, including cancer-specific EVs and tumor-activated platelets.

EVs

EVs are membrane vesicles formed intracellularly and secreted in the extracellular space (58); they are generally distinguished on the basis of their size: small EVs (also called exosomes, 50 to 150 nm in diameter), medium EVs (150 to 300 nm in diameter), and large EVs (between 300 and 1,000 nm in diameter). EVs have different densities and biochemical compositions. Guidelines for classification and techniques for isolation and identification are described in detail on the International Society of Extracellular Vesicles (ISEV) website, https://www.isev.org/.

EVs are thought to originate from endosomes and intraluminal vesicles (ILV) located inside multivesicular bodies. When the early endosomal membrane folds on itself, it tends to sequester proteins, lipids, and cytosol. While part of this cargo is released by late endosomes in lysosomes, some of it can be incorporated into novel generated organelles. These organelles express tetraspanin (CD63) and the membrane proteins LAMP1 and LAMP2, and fuse with the plasma membrane to be released as EVs. The generation of EVs involves the endosomal sorting complex required for transport (ESCRT), which has three components (I, II, and III), and participate in the process of exosome assembly in different cell types. An ESCRT-independent mechanism for exosome generation is also present, as suggested by the evidence that selective inhibitors of ESCRT do not entirely suppress the biogenesis of EVs (59, 60). The heterogeneous cargos in EVs, including lipids, proteins, tumor, mitochondrial DNA (61), and coding along with ncRNAs (62, 63) can vary according to the producing cell and in response to external stimuli (64). These characteristics make them an attractive target for tumor profiling (65). **Table 1** provides a schematic classification of EVs by size, together with a description of their cargo and cellular origin. EVs' roles are not well established, but many studies show their clear biological impact on the recipient cell (66). For example, EVs derived from metastatic cancer cells are able to increase the invasive and migratory potential of the surrounding less aggressive cancer cells (67), while EVs from stromal cells can release ncRNAs into cancer cells and activate intracellular pathways favoring the expansion of therapy-resistant or tumor-initiating cell clones (68).

Despite the growing evidence supporting their role in cancer metastasis and therapy evasion (61, 69), the effects of EVs during the earlier steps of tumorigenesis are less clear. To use EVs as cancer biomarkers, it is important to identify surface markers that could be used for isolation of cancer-derived EVs and standardize the isolation and enrichment techniques. For a better understanding of these issues, we reviewed standard and novel techniques for EV isolation.

EVs: standardized and novel techniques for collection, enrichment, and quantification

The ISEV defined a series of guidelines for isolation, separation concentration, and morphologic and functional characterization of EVs in 2014 and updated them in 2018 (70). **Table 2** summarizes the principal EV isolation techniques, with advantages and disadvantages for each of them. Ultracentrifugation is the most used method and the first option suggested by the ISEV for isolation and concentration of

Table 1. Comparison among three size-based categories of EVs, based on cargo, mechanisms of production, and detection markers.

EVs subtype (70, 71, 77, 78, 79, 80, 81, 82, 83)	Size	Cargo	Origin	Marker
Small EVs	50–150 nm diameter	Lipids, proteins, RNAs, and DNA	Folding of the endosomal membrane into ILVs. Fusion with plasma membrane and release	CD63, CD9, CD81
Medium EVs	150–300 nm diameter	Cytosolic and plasma membrane-associated proteins, cytoskeletal proteins, integrins	Outward budding of plasma membrane	Tetraspanins
Large EVs	300–1,000 nm diameter	Cytosolic and plasma membrane-associated proteins, cytoskeletal proteins, integrins	Outward budding of plasma membrane	Tetraspanins

Abbreviations: CD = cluster differentiation; EV = extracellular vesicle; ILVs = intraluminal vesicles.

EVs from cell culture, biological fluids, and tissues. It consists of a series of centrifugations at high speed for prolonged periods (relative centrifugal force of 100,000 × *g* for at least 2 hours), in sequential steps, that allow the isolation of large, medium, and small EVs. Differential ultracentrifugation, though, is time consuming and can result in a low-purity product. Van Deun and colleagues compared several exosome-isolation techniques from cell culture based on density gradient, ultracentrifugation, and precipitation (71). They found that density gradient–based methodologies resulted in a low yield of high-purity product, whereas the precipitation technique resulted in a high yield of low-purity product (primarily contaminated by serum proteins). Ultracentrifugation resulted in a medium purity and yield when compared with the previous two approaches (71). An example of a technique used to reach higher purity involves a density-gradient floatation approach together with ultracentrifugation (13). Complementary techniques can be adapted to concentrate the EVs further, such as filtration, size exclusion chromatography, and immune isolation (72). Currently, none of these methods is deemed the most efficient, and they should be chosen based on considering the final application of the isolated product.

New techniques, still needing further testing, include tangential-flow filtration (73), field-flow fractionation (74), field-free viscoelastic flow (75), alternating current electrodynamic (*nanoshearing*; ref. 76), acoustics (77), variations of size-exclusion chromatography (78), ion exchange chromatography (79), microfiltration (80), fluorescence-activated sorting (81), deterministic lateral displacement array (82), affinity-based isolation (83), fast protein/high-performance chromatography (84), high-resolution density gradient isolation (Jeppesen, Dennis K et al. "Reassessment of Exosome Composition." Cell vol. 177,2 (2019): 428-445.e18. doi:10.1016/j.cell.2019.02.029), and other microfluidics techniques (85). While these techniques can help obtain purer EVs, obtaining a higher yield of cancer cell–derived EVs has been elusive. Surface marker-based detection techniques, such as fluorescence-activated sorting, could be used sequentially to achieve better results.

Multimarker kits use magnetic beads associated with a cocktail of antibodies against commonly recognized EV markers such as CD9, CD63, or CD81. However, even in this case, high specificity for cancer-derived vesicles is seldom reached. Most of the isolation techniques tend to yield a heterogeneous pellet of EVs, which is derived in great part from non–tumor-related cells. Therefore, a key point in establishing novel isolation techniques must include identifying cancer-specific markers for EVs; while there is likely to be variability according to tumor type, an ideal marker would be stable in an individual or patient cohort, to allow for diagnosis or longitudinal monitoring of the disease.

Non–tumor-derived factors

Tumor-activated platelets: origin and functions

Platelets are anucleate cell fragments produced by megakaryocytes (86), whose activation status and functions are altered as a result of the interactions with tumor cells (87). In human circulating blood,

Table 2. Comparison among major isolation techniques for EVs based on throughput, purity, recovery rate, and needed equipment.

Technique of EVs isolation (58, 63, 64, 69, 70)	Pros	Cons	Application
UC	High sample throughput with relatively high product purity	Time consuming (each centrifugation step is of at least 2 hours) (each cent	Bulk EVs isolation from cell culture media
Precipitation	High sample throughput, and relatively low personnel specialization and machinery needed	Very low product purity	Bulk EVs isolation from cell culture media with subsequent product purification steps
Density gradient based	High product purity	Low sample throughput and appropriate equipment required	Isolation of EVs from body fluids
Size-exclusion chromatography	High product purity	Low sample throughput and appropriate equipment required	Isolation of EVs from body fluids
Tangential flow filtration	High product purity and recovery rate	Low sample throughput and appropriate equipment required	Isolation of EVs from body fluids
Affinity based	High product purity and recovery rate. Highly specificity	Marker dependent	Isolation of EVs from body fluids

Abbreviations: EV = extracellular vesicle; SEC = size-exclusion chromatography; UC = ultracentrifugation.

their count is between 150,000 and 400,000 per microliter (μL), making them a major sampling reservoir. Because of their small size (2–3 μmol/L in diameter; ref. 88), discoid structure, and biophysical characteristics, platelets circulate at the outermost shear fields of flowing blood, in close contact with the vascular endothelium and intravasating cancer cells. This, together with the leakiness of tumor vasculature, enhances tumor biomarker exchange between cancer cells and platelets, resulting in the biological *"activation"* of the latter. One of the most important properties acquired by platelets during this process is the capacity of active uptake and release of biomolecules through an open canalicular system (89–92). This activation process can occur at various stages of cancer development (93). Tumor-activated platelets that survive and recirculate without removal by the liver or spleen, can easily be isolated through blood biopsies. Once isolated, platelets can be analyzed to identify the adaptive changes they went through during cancer development; thus, representing a potentially useful circulating biomarker for cancer diagnosis, staging, and prognostication (94). Cancer cell–derived cytokines can increase platelet production. For example, IL6 enhances thrombopoietin synthesis in liver (95), which results in elevated platelet counts. Moreover, cancer cells recruit platelets to tumor niches and activate them via factors like TGFβ, ADP, and CD24; consequently, platelets trigger proangiogenic and proinflammatory stimuli in the tumor microenvironment (96). Platelet surface activation markers, such as activated α2b-β3, lysosomal-activated membrane protein (CD63), and P-selectin (CD62P) can be easily detected via flow cytometry together with platelet-specific antigens such as glycoprotein complex Ib-IX-V, GPIIb (CD41), and GPIIIa (CD61; ref. 97).

Tumor-activated platelet profiling

As for platelet-derived markers, researchers have lately focused greatly on RNA profiling. Despite being anucleate cell derivatives, platelets contain several types of RNAs, including messenger RNAs (precursor-mRNA and mature mRNA), structural and catalytic RNAs (rRNA, transfer RNA, and small nucleolar RNA), and regulatory RNAs (miRNA, long intergenic ncRNA, pseudogenes, and antisense RNA). Platelets can also translate mRNA into proteins (98) and splice mRNAs under external stimulation (99), to produce a unique array of mRNAs (100). Amplification and sequencing of platelet-derived mRNA in many patients with localized and metastatic cancer (NSCLC, colorectal, glioblastoma, pancreatic, hepatobiliary, and breast) have revealed the existence of a specific RNA profile present in patients with cancer (94). These mRNAs with increased expression in tumor-activated platelets were enriched for processes associated with vesicle-mediated transport and cytoskeletal protein binding. Diagnostic accuracy for this platelet-based pan-cancer classification reached an accuracy of 95% in distinguishing patients from healthy controls and 97% in predicting different tumor types. Two more recent studies focusing on breast cancer (101) and glioblastoma (102), confirmed the existence of different specific TEP-RNA-seq signatures in tumor patients versus healthy controls. Additional studies are needed to assess the stability of these mRNA signatures in several patient cohorts.

Tumor-activated platelet metabolism

Platelet metabolism is also influenced by interaction with cancer cells; in fact, platelet activation relies on metabolic switches that often involves lipid metabolism toward prostaglandins, prostacyclin, and thromboxane production. These components have a short half-life (seconds to a few minutes), and their synthesis can be influenced by external factors; proinflammatory stimuli tend to mobilize the membrane-bound arachidonic acid, which is used to synthesize bioactive lipids via the COX enzymes (103). Prostaglandins, in turn, favor tumorigenesis via several mechanisms, the most well-known being the modulation of the β-catenin pathway in colon cancer (104). Detection of metabolic profiles of tumor-activated platelets is therefore of great interest in liquid biopsy–based biomarker research. Platelet metabolomics has been extensively studied in the cardiovascular field, using nuclear magnetic resonance (105, 106). This technique could overcome some of the limitations of RNA sequencing, represented by the high cost and the complex interpretation of results.

When analyzing the content of platelets, it is extremely important to preemptively adopt an isolation technique that guarantees high purity of yield. White and red blood cells are common contaminants of the platelet-eluate and can significantly alter the quantity and quality of RNA and metabolites. For a better understanding of these issues, we review in the following paragraph, the major steps involved in isolation of platelets and some of the commonly used analytic techniques.

Tumor-activated platelets: standardized and novel techniques for isolation and morphologic analysis

The first well-established step for isolation of platelets consists of centrifugation of whole blood (6 minutes, 1,100 rpm) collected in a propylene tube containing acid citrate dextrose or sodium citrate (107). The product is a three-layered solution containing platelet-rich plasma (PRP) on top, a thin layer of white blood cells called the buffy coat, and red blood cells at the bottom. After PRP is collected, prostaglandin I2 (PGI2, prostacyclin) or *EDTA* is usually added [at a concentration of 0.5 μmol/L/L for the former (108) and 5 mmol/L for the latter], to avoid platelet activation. Subsequently, a series of differential centrifugation steps is performed to enrich the product for platelets. This step can be affected by the occurrence of white and red blood cell contamination and also by exposure to activating stimuli. Therefore, size-exclusion approaches such as elution on a sepharose column (109) are often adopted as an alternative to the sequential centrifugations. Microfluidics platforms exploit the small size of a single platelet (2–3 μmol/L in diameter) and can be combined with a second affinity-based technique such as acoustics (110) to achieve higher purity.

Isolated platelets can be analyzed by count, function, and morphology. Usually, functional tests assess platelet aggregation (*in vitro* platelet-to-platelet aggregate formation via GPIIb/IIIa), platelet *adhesion* (*in vitro* ability of platelets to adhere under shear conditions), and platelet *activation* (flow cytometry analyzes surface and intracellular activation markers). The study of tumor-activated platelets often requires both functional and morphologic analyses; while functional analyses are well standardized (111), the morphologic analyses are conducted mostly using experimental techniques.

Platelets are normally round or oval when in EDTA (Ethylenediaminetetraacetic acid) anticoagulant; upon activation, they develop a characteristic star-shaped appearance, where the central part is occupied by scattered granules, which appear azurophilic (112). The interaction with cancer cells results in an alteration in size, granule localization, mitochondria number and localization, and surface pseudopodia development. Wang and colleagues (113) reported a characteristic change in microtubule and granule distribution in circulating platelets from patients with ovarian cancer as compared with platelets from matched controls. This analysis relied on electron cryotomography, which allows for tridimensional imaging via the acquisition of multiple imaging stacks, resulting in a sharp image, avoiding sample fixation.

Platelet imaging should encompass high-resolution techniques such as structured illumination microscopy and single-molecule localization microscopy, which allow in depth analyses of the cytoskeleton and

the actin nodules. Recent advances in multiplexing, with the development of multiplexed flow cytometry and mass cytometry able to target a high number of antigens at once, will more suitably serve platelet morphology studies (114, 115).

A single biomarker is not able to adequately detect and monitor tumors (116–120). The collection of large amounts of complex data represented by tumor tissue–based *OMICS*, liquid biopsies-derived DNA-sequencing, proteomics, and EVs and platelets will require artificial intelligence tools for integration and analysis (121–123). An attempt in this direction resulted in the generation of *PlateletWeb*, a platform that integrates literature data with available bioinformatics databases (such as the Uman Protein Database and Gene Ontology) on proteome and transcriptome, to provide a novel tool for the analysis of platelet signaling.

Conclusions

The increased number of targeted therapies available and the need to use them earlier during patients' clinical course make diagnostic and prognostic protocols increasingly more important. Tumor biopsies are essential to diagnose cancer and provide helpful guidance to clinical practice. Nonetheless, they are limited in conveying all the necessary information due to biological and technical limitations. Liquid biopsies exploit the ability of cancer cells to release biological mediators that can influence the activity of other cells. These mediators can be detected and analyzed to provide information about tumor composition, metastatic behavior, and therapy sensitivity. Moreover, being minimally invasive, they can be repeated longitudinally with a higher level of compliance by patients.

CTCs and cfDNA have been used successfully in several prognostic and predictive clinical studies; moreover, novel strategies using EVs and tumor-activated platelets are being tested. Although these new technologies hold promise for clinical applications, they cannot fully substitute imaging and tumor tissue analysis; however, they can complement them. Because the biggest drawback of liquid biopsy is the low concentration of available cancer-derived material, it will be important to increase the specificity of isolation and enrichment techniques while maintaining their accessibility and cost-effectiveness.

The FDA-approved liquid biopsy–based tests primarily have two applications; these include as a companion diagnostic (FoundationOne and Guardant360) and predictor of outcome (Cell Search). The ct/DNA tests also provide insights regarding the genetic make-up of the tumor to guide targeted therapies. Nonetheless, these applications are biased on one side by the reduced specificity of the circulating cells captured by the assay and on the other by the potential confounding factor from hematopoietic precursors bearing clonal mutation (described above).

We, therefore, envision novel clinical applications for the newly developed liquid biopsy markers (circulating EVs and platelets), including potential for early diagnosis of individuals at risk for developing cancer (e.g., those with BRCA mutation). Clinical trials could benefit from the inclusion of liquid biopsy–based measurements, both at diagnosis and during treatment, ideally at multiple timepoints. Moreover, regular measurements for patients during follow-up or for healthy individuals at risk could be implemented. We recognize that the absence of a specific cancer-derived EV marker and the relatively short lifespan of platelets represent important limitations for their use in clinical practice; these and other assays will require further development and validation studies. Finally, merging multiple datasets to identify informative patterns is the ultimate step in identifying diagnostic, predictive, and prognostic biomarkers to be validated and approved for routine clinical use.

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